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EP 98/8563

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Dated 25 November 1998

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24 DEC 1997
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P01/7700 25.00 - 9727262.9

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9727262.9

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# Request for grant of a Patent

Form 1/77 **Patents Act 1977**

## ❶ Title of invention

1 Please give the title of the invention: VACCINE

## ❷ Applicant's details

☐ **First or only applicant**

2a If you are applying as a corporate body please give:
Corporate Name: SmithKline Beecham Biologicals SA

Country (*and State of incorporation, if appropriate*): Belgium

2b If you are applying as an individual or one of a partnership please give in full:

Surname

Forenames

2c **In all cases,** please given the following details:

Address: Rue de l'institut 89, B-1330 Rixensart

UK postcode *(if applicable)*

Country: Belgium

ADP number *(if known)*: ~~5800974002~~ 05781170201

*2d, 2e and 2f: If there are further applicants please provide details on a separate sheet of paper*

## ❑ Second applicant *(if any)*

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## ❸ Address for service details

3a Have you appointed an agent to deal with your application?
Yes ☒ No ☐ ➲ *go to 3b*
⓫
***please give details below***

Agent's name MARCUS J W DALTON

Agent's address SmithKline Beecham
Corporate Intellectual Property
New Horizons Court
Great West Road
Brentford, Middlesex

Postcode TW8 9EP

Agent's ADP number 041223130103

**3b:** *If you have appointed an agent, all correspondence concerning your application will be sent to the agent's United Kingdom address.*

3b If you have not appointed an agent please give a name and address in the United Kingdom to which all correspondence will be sent:

Name

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## ❹ Reference number

4. Agent's or applicant's reference number *(if applicable)* MJWD/B45124

## ❺ Claiming an earlier application date

5. Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

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Yes ☐ No ☒ ➲ *go to 6*

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## ❻ Declaration of priority

*If you are declaring priority from a PCT Application please enter 'PCT' as the country and enter the country code (for example, GB) as part of the application number*

*Please give the date in all number format, for example, 31/05/90 for 31 May 1990*

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|---|---|---|
| | | |

❼

*any applicant is not an inventor*
- *there is an inventor who is not*
- *an applicant, or*
- *any applicant is a corporate body.*

## ❼ Inventorship

7. Are you (the applicant or applicants) the sole inventor or the joint inventor

*Please mark correct box*

Yes ☐ No ☒ ➲ ***A Statement of Inventorship on Patents form 7/77 will need to be filed (see Rule 15).).***

❽ *Please supply duplicates of claim(s), abstract, description and drawings).*

## ❽ Checklist

8a Please fill in the number of sheets for each of the following types of document contained in this application

Continuation sheets for this Patents Form 1/77 [ – ]

Claim(s) [ ] Description [ 17 ]

Abstract [ ] Drawing(s) [ ]

8b Which of the following documents also accompanies the application?

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Priority documents *(please state how many)* [ – ]

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Patents Form 7/77 - Statement of Inventorship and Right to Grant [ – ]

Patents Form 9/77 - Preliminary Examination Report [ – ]

Patents Form 10/77 - Request for Substantive Examination [ – ]

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## ❾ Request

I/We request the grant of a patent on the basis of this application.

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Signed Marcus J.W. Dalton Date: 24/12/97

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Chartered Patent Attorney

Attorney for the Applicant

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# Vaccine

The present invention relates to new adjuvants, and to pharmaceutical compositions containing them, and to the use of such adjuvants compositions in medicine.

Immunomodulatory oligonucleotides containing unmethylated CpG dinucletodies ("CpG") are known (WO 96/02555, EP 468520). Such oligonucleotides can activate various immune subsets, including natural killer cells (which produce interferon γ and have cytolytic activity) and macrophages (Wooldridge et al Vol 89 (No. 8), 1977).

The present inventors have found that such oligonucleotides when formulated with suitable antigens produce a potent vaccine formulation. A preferred oligonucleotides has the following sequence:

OLIGO 2: TCT CCC AGC GTG CGC CAT

One of the vaccine such oligonucleotides the present inventors have shown to be effective is in the prophylasix and treatment of Papillomvirus infections.

Papillomaviruses are small naked DNA tumour viruses (7.9 kilobases, double strand), which are highly species-specific. Over 70 individual human papillomavirus (HPV) genotypes have been described. Papillomaviruses are classified on the basis of species of origin (human, bovine etc.) and of the degree of genetic relatedness with other papillomaviruses from the same species. HPVs are generally specific for the skin or mucosal surfaces and usually cause benign tumours (warts) that persist for several months or years. Some HPVs are associated with cancer. The strongest positive association between an HPV virus and human cancer is that which exist between HPV 16 and 18 and cervical carcinoma. More than ten other HPV types have also been found in cervical carcinomas although at less frequency.

Genital HPV infection in young sexually active women is common and most individuals either clear the infection, or if lesions develop, these regress. Only a subset of infected individuals has lesions which progress to high grade intraepithelial neoplasia and only a fraction of these progress further to invasive carcinoma.

The molecular events leading to HPV infection have not been clearly established. The lack of an adequate *in vitro* system to propagate human papillomaviruses has hampered the progress to a best information about the viral cycle.

Today, the different types of HPVs have been isolated and characterised with the help of cloning systems in bacteria and more recently by PCR amplification. The molecular organisation of the HPV genomes has been defined on a comparative basis with that of the well characterised bovine papillomavirus type 1 (BPV1).

Although minor variations do occur, all HPVs genomes described have at least seven early genes, E1 to E7 and two late genes L1 and L2. In addition, an upstream regulatory region harbors the regulatory sequences which appears to control most transcriptional events of the HPV genome.

E1 and E2 genes are involved in viral replication and transcriptional control, respectively and tend to be disrupted by viral integration. E6 and E7, and recent evidence implicate also E5 are involved in viral transformation.

In the HPVs involved in cervical carcinoma such as HPV 16 and 18, the oncogenic process starts after integration of viral DNA. The integration results in the inactivation of genes coding for the capsid proteins L1 and L2 and in installing continuously over expression of the two early proteins E6 and E7 that will lead to gradually loss of the normal cellular differentiation and the development of the carcinoma.

Carcinoma of the cervix is common in women and develops through a pre-cancerous intermediate stage to the invasive carcinoma which frequently leads to death. The intermediate stages of the disease is known as cervical intraepithelial neoplasia and is graded I to III in terms of increasing severity.

Clinically, HPV infection of the female anogenital tract manifests as cervical flat condylomas, the hallmark of which is the koilocytosis affecting predominantly the superficial and intermediate cells of the cervical squamous epithelium.

Koilocytes which are the consequence of a cytopathic effect of the virus, appear as multinucleated cells with a perinuclear clear haloe. The epithelium is

thickened with abnormal keratinisation responsible for the warty appearance of the lesion.

Such flat condylomas when positive for the HPV 16 or 18 serotypes, are high-risk factors for the evolution toward cervical intraepithelial neoplasia (CIN) and carcinoma in situ (CIS) which are themselves regarded as precursor lesions of invasive cervix carcinoma.

International Patent Application No. WO 96/19496 discloses variants of human papilloma virus E6 and E7 proteins, particularly fusion proteins of E6/E7 with a deletion in both the E6 and E7 proteins. These deletion fusion proteins are said to be immunogenic.

The present invention provides compositions comprising either an E6 or E7 protein linked to an immunological fusion partner having T cell epitopes, the composition being further adjuvanted with a CpG containing oligonucleotide.

In a preferred form of the invention, the immunological fusion partner is derived from protein D of Heamophilus influenza B. Preferably the protein D derivative comprises approximately the first 1/3 of the protein, in particular approximately the first N-terminal 100-110 amino acids.

Accordingly, the present invention in the embodiment provides fusion proteins comprising Protein D - E6 from HPV 16, Protein D - E7 from HPV 16 Protein D - E7 from HPV 18 and Protein D - E6 from HPV 18. The protein D part preferably comprises the first 1/3 of protein D.

The proteins of the present invention preferably are expressed in E. coli. In a preferred embodiment the proteins are expressed with a Histidine tail comprising between 5 to 9 and preferably six Histidine residues. These are advantageous in aiding purification. The description of the manufacture of such proteins is fully described in co-pending UK patent application number GB 9717953.5.

The oligonucleotides are typically deoxynucleotides. In a preferred embodiment the internucleotide bond in the oligonucletodie is phosphorodithioate, or more preferably phosphorothioate bond, although phosphodiesters are within the scope of the present invention.

In a preferred embodiment CpG oligonucleotides are utilised to adjuvant a vaccine for the prevention of HPV infections.

Such vaccines are advantageous in that an enhanced level of cell mediated immunity is provided as compared to other adjuvant formulations. This formulation had a significant effect in the treatment of TC1 induced tumours, resulting in tumour regression, with CD8 infiltrating the tumour, and CTL in the spleen.

In an another embodiment of the invention, polysaccaharide based vaccine are provided. In particular there is provided a Streptococcus pneumoniae vaccine adjuvanted with a CpG oligodeoxynucleotide as described herein. Preferably the vaccine is formulated with the oligonucleotide designated oligo 2 above.

Polysaccharide antigen based vaccines are well known in the art, and are typically directed to the prevention of meningocococcal and pneumococcal vaccines. However, polysaccharide vaccines are poor immunogens themselves and to be effective vaccines conjugation or covalent bonding to an appropriate protein carrier (eg Tetanus Toxoid or Diptheria Toxoid) has been required to enable T-cell response to be elicited. Commercially available vaccine based on such conjugate technology are available to prevent Heamophilius influenzae infections and Streptococcus pneumoniae.

Surprisingly, the present inventors have found that it is possible adjuvant the immune response to unconjugated streptococcus pnemonia polysaccaharide vaccines by formulating with CpG oligonucleotide, such formulations provided an immune response equivlanet to conjugated vaccines.

When combined with a commercially available 23 valent conjugated polysaccaharide vaccine (Pneomovax, Pasteur Merieux), CpG adjuvantion significantly augmented the immune response ( IgG antibody) especially to types 19F and 14.

The present invention also provides vaccine formulations comprising a CpG oligonucleotide preferably formulated with Oligonucleotide 2 with other antigens or antigenic compositions which include for example, antigen or antigenic compositions derived from HIV-1, (such as gp120 or gp160), any of Feline Immunodeficiency virus, human or animal herpes viruses, such as gD or derivatives

thereof or Immediate Early protein such as ICP27 from HSV1 of HSV2, cytomegalovirus (especially human) (such as gB or derivatives thereof), Varicella Zoster Virus (such as gpI, II or III), from other viral pathogens, such as Respiratory Syncytial virus (for example RSV F and G proteins or immunogenic fragments thereof disclosed in US Patent 5,149,650 or chimeric polypeptides containing immunogenic fragments from HRSV proteins F and G, eg GF glycoprotein disclosed in US Patent 5,194,595), antigens derived from meningitis strains such as meningitis A, B and C, human papilloma virus, in particular from strains HPV6, 11, 16 and 18, Influenza virus, Haemophilus Influenza B (Hib), Epstein Barr Virus (EBV), or derived from bacterial pathogens such as Salmonella, Neisseria, Borrelia (for example OspA or OspB or derivatives thereof), or Chlamydia, or Bordetella for example P.69, PT and FHA, or derived from parasites such as plasmodium or toxoplasma.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al, University Park Press, Baltimore, Maryland, USA 1978. Encapsulation within liposomes is described, for example, by Fullerton, US Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, US Patent 4,372,945 and by Armor et al, US Patent 4,474,757.

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-1000 μg of protein, preferably 2-100 μg, most preferably 4-40 μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initital vaccination, subjects may receive one or several booster immunisation adequately spaced.

The CpG oligonucleotides utilised in the present invention may be synthesized by any method known in the art (eg EP 468 520) conveniently such oligonucleotides can be synthesized utilising an automated sequencer. Methods for

producing phosphorthioate oligonucleotides or phosphorodithioate are described in US 5,663,153, US 5,278,302 and WO 95/26204.

The following example serves to illustrate the present invention.

**Example 1:**

The therapeutic potential of vaccine containing two different CpG oligonucleotides or 3D-MPL or 3D-MPL/QS21/Cholesterol (3D-MPL/DQ) and 3D-MPL + alum or 3D-MPL + CpG was evaluated in the TC1, E7 expressing tumour model.

**1.1 Therapeutic experiments: protocol**

10e6 TC1 cells, E7 expressing tumour cells: were injected subcutaneously (200µl) in the flank of C57BL/6 immunocompetent mice. Mice were vaccinated 7 and 14 days after the tumour challenge, with 5µg ProtD 1/3 E7 HPV16 injected intra-footpad (100µl : 50µl / footpad) in the presence of different adjuvants: (a) 3D-MPL, (b) 3D-MPL, QS21-Cholesterol (DQ), (c) 3D-MPL alum, (d) CpG oligos 1+ 3D-MPL + oligo 1.

10 animals/group

2 and 4 weeks after the second immunisation, 5 mice/group were killed and spleens or popliteal lymph nodes were taken and pooled.

| EXPERIMENTS | IMMUNISATIONS | ORGAN/ HARVESTING | Lot Prot D1/3E7 | stc |
|---|---|---|---|---|
| CGHPV97010/LAS97293 | day 7, 14 | day 28, 42 | 957/015' | 67 |

The **tumour growth** was monitored by measuring individual tumours twice a week.

**Antibody response** to E7 was monitored by ELISA on the serum 2 weeks post II.

**Lymphoproliferative response** was analysed by *in vitro* restimulation of spleen and lympth nodes cells for 72 hrs with either PD1/3E7, the protein E7(Bollen) and PD (whole) PD1/3 (coated or not on latex µbeads) (10, 1, 0.1 µg/ml) 2 and 4 weeks post II.

**Cytokine production** was measured by ELISPOT after 96 hours of *in vitro* re-stimulation with the E7 protein (Bollen) 2μg/ml.

**CTL response** was measured 2 and 4 weeks podt II, after *in vitro* re-stimulation of spleen cells with irradiated tumour cells (TC1) in the presence or not of conA supernatant.

The Chromium release assay was performed on TC1 cells, on a syngeneic tumour cell line: EL4 pulsed or not with an E7-derived and on K562 cells a target for NK mediated lysis.

**1.2 Results**

**Groups of mice**

1) PBS
2) ProtD1/3 E7 HPV16
3) ProtD1/3 E7 HPV16 + 3D-MPL, QS21/Cholesterol (DQ)
4) ProtD1/3 E7 HPV16 + o/w emulsion/3D-MPL/QS21
5) ProtD1/3 E7 HPV16 + oligo 1: 1826 (WD 1001): TCC ATG ACG TTC CTG ACG
6) Oligo 1
7) ProtD1/3 E7 HPV16 + oligo 2/ 1758 (WD1002): TCT CCC AGC GTG CGC CAT
8) Oligo 2
9) ProtD1/3 E7 HPV16 + 3D-MPL
10) ProtD1/3 E7 HPV16 + 3D-MPL + oligo 1

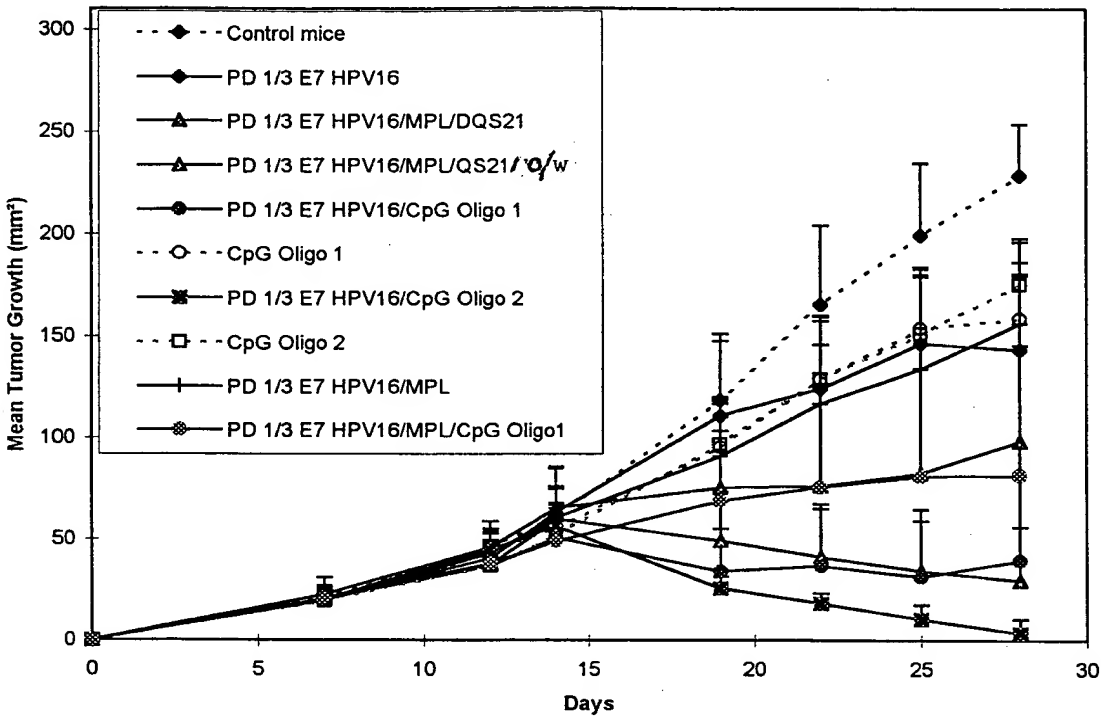
**Tumour Growth;**

(The graphic represents the mean tumour growth (in mm2)/group n=10 followed over 4 weeks)

Day 28

5

LAS97293/CGHPV97010




The injection of 10e6 TC1 cells injected subcutaneously give rise to a growing tumour in 100% of the animals.

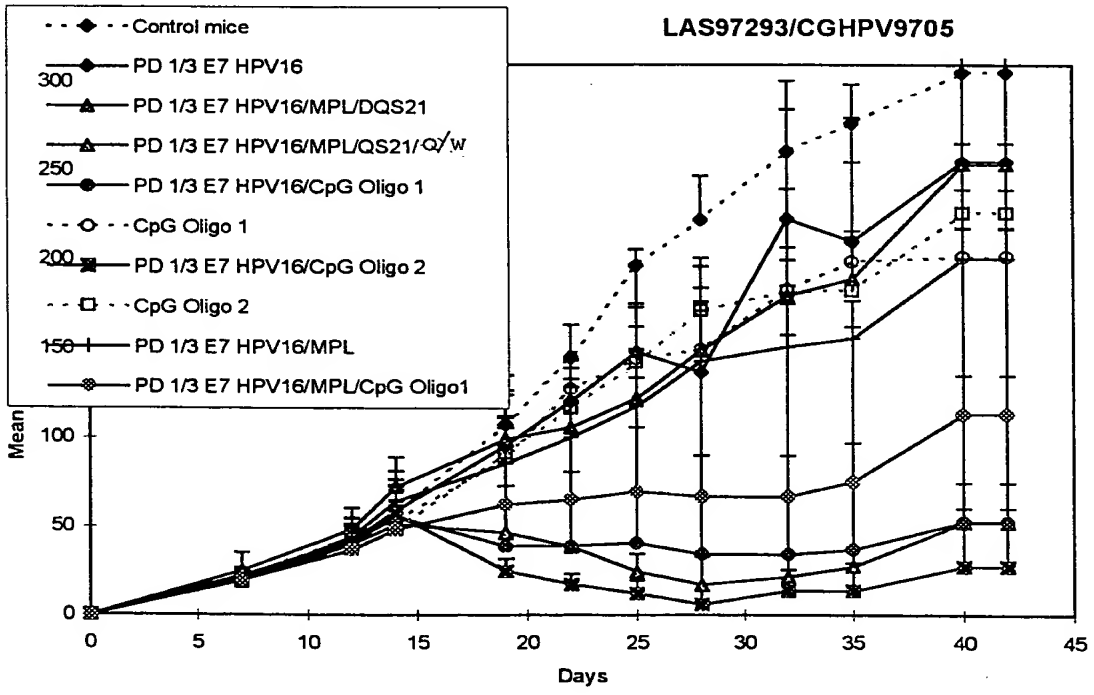
Vaccinating with ProtD1/3E7 or adjuvant alone: 100% of the animals develop a tumour.

10 As shown on the graphic, in the groups of mice that received the antigen with a CpG oligonucleotide the mean tumour growth remained very low and very similar between groups, reflecting that the tumour growth either was slowed down or that several tumours were completely rejected.

The analysis of individual tumour growth 2 and 4 weeks after the latest vaccination showed that complete rejection in the groups were:

| | Day 28 (n=10) | day 42 (n=5) |
|---|---|---|
| E7+alum+3D-MPL | 20% | 0% |
| **E7+oligo1 (1826)** | **40%** | **40%** |
| Oligo1 | 0% | 0% |
| **E7+oligo2 (1758)** | **70%** | **40%** |
| Oligo2 | 0% | 0% |
| E7+3D-MPL | 0% | 0% |
| E7+3D-MPL+Oligo1 | 20% | 0% |

**Day 42**




(The graphic represents the mean tumour growth (in mm2/group n=5 followed over 6 weeks).

The mean tumour growth/group of mice vaccinated with PD1/3 E7+ the CpG oligos or the 3 D- MPL QS21 O/W formulation are quite similar, but again the analysis of the individual tumour growth showed that the CpG oligos induce more prolonged complete tumour rejection (0% with O/W formulation versus 40% with Oligos).

**Conclusion**

Both CpG (Oligo 2 > oligo 1) induced more complete tumour regression than 3D-MPL/QS21/o/w emulsion.

**Proliferative responses**

Positive controls (ConA stimualtion) were positive.

Surprisingly, no E7 specific and no PD specific proliferative response could be observed starting with spleen cells 2 or 4 weeks post II (probably due to a technical problem: data not shown).

On the contrary, lymph node cells from mice that received ProtD1/3 E7 in 3D-MPL + QS21/o/w emulsion or CpG oligos 1 and 2 showed a very good E7 specific proliferative response although almost no PD (whole) specific response could be observed even at the hightest concentration of 100μg/ml no PD1/3 specific responses was observed even when coated on latex μbeads.

Similar data were obtained 4 weeks post II.

**Serology**

The anti E7 antibody response: IgG tot and isotypes (IgG1, IgG2a, IgG2b, IgGTot) were measured by ELISA using the E7 protein (Bollen) as coating antigen as described in the Materials and Methods. The figure show the relative percentage of the different IgG isotypes in the total of IgGs, 2 and 4 weeks post II.

The weak antibody response induced after 2 vaccinations with the ProtD1/3 E7 3D-MPL/QS21 o/w emulsion was strongly increased in animals that received the protein with an adjuvant 3D-MPL, QS21, Cholesterol.

3D-MPL did not induce a strong antibody response.

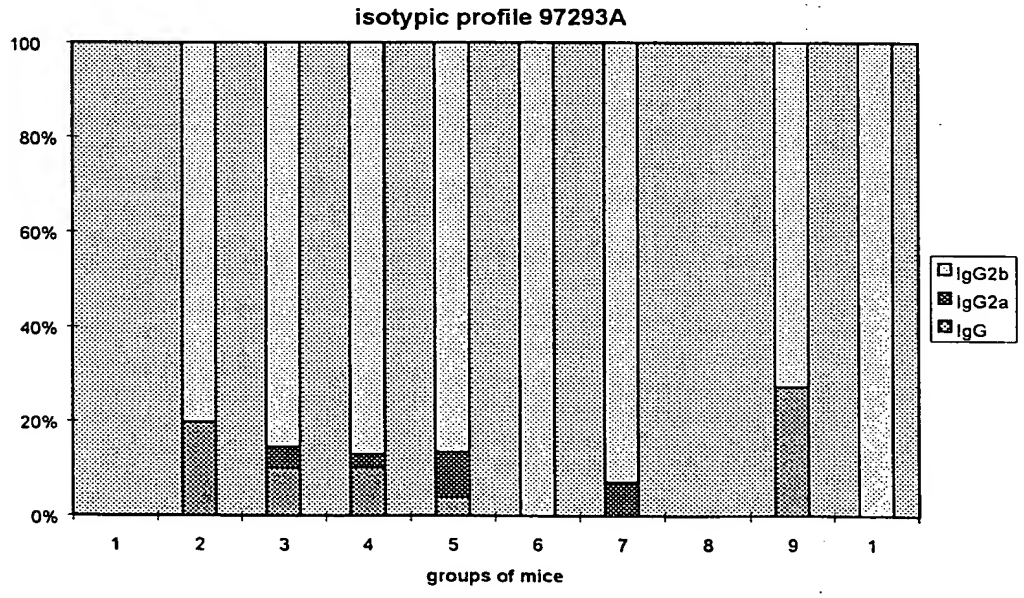
The Oligos affect only weakly (oligo 2) or not at all (Oligo 1) the weak antibody response observed when PD1/3E7 alone was injected.

The predominant E7 specific antibody subclass was clearly IgG2b for all the formulation tested (80-90% of the total IgGs).

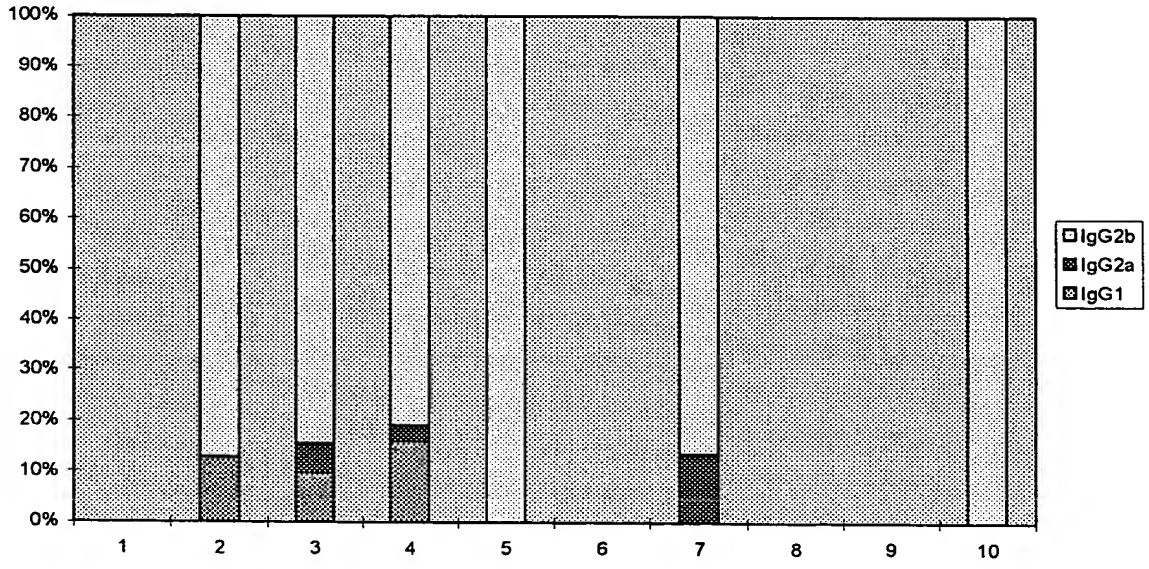
Same results were obtained 4 weeks post II (data not shown).

**Isotypic profile of anti E7 responses (post II, pooled sera) exp. 97293**

| Groups | IgG1 | IgG2a | IgG2b | IgGtot |
|---|---|---|---|---|
| 1) PBS | 0 | 0 | 0 | 0 |
| 2) ProtD1/3 E7 HPV16 | 1020 | 0 | 4130 | 4740 |
| 3) ProtD1/3 E7 HPV16 + QS21/3D-MPL/ Cholesterol | 4720 | 2050 | 39960 | 64280 |
| 4) ProtD1/3 E7 HPV16 + QS21/3D-MPL/o/w emulsion | 7290 | 1840 | 61310 | 61620 |
| 5) ProtD1/3 E7 HPV16 + oligo 1 | 170 | 400 | 3680 | 4910 |
| 6) Oligo 1 | 0 | 0 | 530 | 420 |
| 7) ProtD1/3 E7 HPV16 + oligo 2 | 0 | 590 | 7560 | 13690 |
| 8) Oligo 2 | 0 | 0 | 0 | 0 |
| 9) ProtD1/3 E7 HPV16 + 3D-MPL | 1380 | 0 | 3670 | 4710 |
| 10) ProtD1/3 E7 HPV16+ 3D-MPL + oligo 1 | 0 | 0 | 8950 | 9900 |



| Groups | IgG1 | IgG2a | IgG2b | IgGtot |
|---|---|---|---|---|
| 1) PBS | 0 | 0 | 0 | 0 |
| 2) ProtD1/3 E7 HPV1 | 240 | 0 | 1650 | 1400 |
| 3) ProtD1/3 E7 HPV16 + QS21/3D-MPL/ Cholesterol | 1420 | 850 | 12460 | 13480 |
| 4) ProtD1/3 E7 HPV16 + 3D-MPL QS21/o/w emulsion | 5880 | 1130 | 29690 | 28340 |
| 5) ProtD1/3 E7 HPV16 + oligo 1 | 0 | 0 | 1280 | 1430 |
| 6) Oligo 1 | 0 | 0 | 0 | 0 |
| 7) ProtD1/3 E7 HPV16 + oligo 2 | 0 | 560 | 3600 | 5880 |
| 8) Oligo 2 | 0 | 0 | 0 | 0 |
| 9) ProtD1/3 E7 HPV16 + 3D-MPL | 0 | 0 | 0 | 490 |
| 10) ProtD1/3 E7 HPV16+ 3D-MPL + oligo 1 | 0 | 0 | 830 | 1180 |




**CTL assay:**

A CTL response could be detected when measured 2 weeks after the latest vaccination, when cells were re-stimulated in vitro with irradiated TC1 when TC1 or peptide E7 pulsed EL4, were used as target cells when mice immunised with PD1/3 E7 + CpG oligo 2 > 1 (25-40% specific lysis) and not with oligos alone.

No CTL response could be measured when QS21/3D-MPL/o/w emulsion + antigen was used (contrarily to what we observed previously (memo HPV ther2).

QS21/3D-MPL Cholesterol only showed a weak CTL response upon re-stimulation with irradiated TC1 cells and ConA supernatant.

A better lysis was seen on TC1 cells than on peptide E7 pulsed EL4 cells, but this is mostly observed in the groups of mice vaccinated with PD1/3E7 + CpG oligos (2 > 1). In this experiment other formulations did not induce a CTL response although 3D-MPL/QS21/oil in water emulsion induced nice tumour regression.

Using E7 pulsed EL4 cells, no lysis was observed when mice received the protein or the adjuvant alone.

**Conclusions**

CpG containing oligos have a potent adjuvant effect in the treatment of pre-established TC1 tumours.

Cell mediated immunity is clearly enhanced by CpG (antigen specific lymphoproliferative and CTL response).

CpG oligos in this model only marginally affected the antigen specific antibody response.

### 1.3 Materials and Methods

| Component | Brand | Batch number | Concentration (mg/ml) | Buffer |
|---|---|---|---|---|
| ProtD1/3-E7 | | 957/015 | 0.677 | PBS 7.4 |
| MPL | Ribi | 040 | 2.723 | $H_20$ |
| QS21 | Aquila | A7030R | 2 | $H_20$ |
| oligo CpG 1826 | EuroGentec | WD1001 | 5 | $H_20$ |
| oligo CpG | EuroGentec | WD1002 | 5 | $H_20$ |
| Liposomes | | SUV002 | 40 DOPC<br>10 Chol | PBS 7.4 |
| o/w emulsion | SB | ESB62010 | 2x | PBS 6.8 |

#### 1.3.1 Formulation Process

All the formulations were prepared on the day of injection.

**QS21/3D-MPL or MPL based formulations**

ProtD1/3-E7 antigen (5μg) was diluted in 10 fold concentrated PBS pH 7.4 and $H_20$ before addition of MPL (5μg). If needed, after 30 minutes, QS21 (5μg) was added to the formulation, mixed with liposomes in a weight ratio QS21/cholesterol of 1/5 (referred to as DQ). If needed, 100μg of CpG oligonucleotide were added 30 minutes later DQ addition. All incubations were carried out at room temperature with agitation. A detailed description of such DQ formulations can be found in International Patent patent application number WO 96/33739.

**Oligo containing formulations**

Formulations containing oligo alone without other adjuvant were prepared by addition of CpG to the diluted PrtD1/3-E7 in PBS pH 7.4.

**Oil in Water based formulations**

ProtD1/3-E7 (5μg) was diluted in 10 fold concentrated PBS pH 6.8 and $H_20$ before consecutive additon of the emulsion, MPL (5μg) and QS21 (5μg) at 5 minute interval. All incubations were carried out at room temperature with agitation. The preparation of the emulsion is described in International patent application WO 95/17210.

The adjuvant controls without antigen were prepared by replacing the protein by PBS.

**1.3.2 Mice and Cell lines**

**Mice** C57Bl/6 (Iffa Credo) 6-8 weeks old mice were used in these experiments.

**Cell lines**: TC1 (obtained from the John Hopkin's University) , or EL4 cells were grown in RPMI 1640 (Bio Whittaker) containig 10% FCS and additives: 2mM L-Glutamine , 1% antibiotics (10000U/ml penicilin, 10000μg/ml streptomycin) 1% non essential amino acid 100x, 1% sodium pyruvate (Gibco), 5 10e-5 M 2-mercaptoethanol. Before injection TC1 cells were trypsynized and washed in serum free medium.

**1.3.3 Tumour growth:**

All the animals were injected with tumor cells on day O and were randomized at day 7. Individual tumor growth was followed over time ( the 2 main diameters ( A, B) were measured using calipers twice a week, A x B represents the "tumor surface" and the average of the 5 values / groups is showed on a graphic over time: 6 weeks

**1.3.4 CMI read out**

**In vitro lymphoproliferation**

Lymphoproliferation was performed on individual spleens and on lymph node pools. 200000 spleen cells or popliteal lymph node cells were plated in triplicate, in 96 well microplate, in RPMI medium containing 1% normal mice serum and additives . After 72 hrs of in vitro re-stimulation with different amounts of PD1/3 E7 (1, 0.1, 0.01 μg/ml) or E7 ( Bollen ) ( 10-1-0.1 μg/ml ) After 72hrs, 100 μl of culture supernatant were removed and replaced by fresh medium containing 1μCi 3H thymidine (Amersham 5Ci/mmol) . After 16 hrs, cells were harvested onto filter plates. Incorporated radioactivity was counted in a β counter. Results are expressed in CPM (mean of triplicate wells) or as stimulation indexes (mean CPM in cultures with antigen / mean CPM in cultures without antigen).

**Cytokine production (elispot protocol cfr Phe)**

Cell suspensions were prepared in RPMI 1640 medium (GIBCO) containing 2 mM L-glutamine, antibiotics, 5x10-5 M 2-mercaptoethanol, and 5% foetal calf serum. Three cells dilutions (10e5, 2 10e4, 4 10e3) were seeded in triplicate (100μl/w in flat-bottomed 96 well- plates pre-coated with 5μg/ml PBS purified MAb to cytokine (IL4 (Pharmingen 18031D), IL5 (Pharmingen 19241W), IL10 (Pharmingen 18141D), IFNg (Genzyme, 80.3955.02) 50μl/w, and saturated with RPMI medium containing 10% FCS and antibiotics for 1Hr at 37°C 200μl/w . 10e5 naïve irrradiated( 2000r ) splenocytes were added to the 2 latest cell dilutions.

Cells were stimulated with 2 μg /ml of E7 protein. (Bollen) for 96H at 37°C Controls were feeder cells + antigen or 10e5 effector cells without antigen.

Plates were then manually wahed with PBS Tween 0.2% . Biotinylated antibodies to cytokine ( anti IL4 (Pharmingen 18042D, IL5 (Pharmingen 19062D),

IL10 (Pharmingen 18152D), IFNg (Genzyme 80395702) were added at 1µg/ml in PBS BSA 1% Tween2O 0.1% FCS 4% (50µl/w) for 1H 30 at 37°C

After washing, 1000X diluted anti biotin goat Abs, conjugated to gold particles (BritishBioteCell International, GAB1) was added for 1Hr at 37°C after another whash, revelation was performed using the Silver enhancing Kit (BBI ) add demineralized water to stop the reaction after 45 min at room temperature.

**1.3.5 CTL assay**

20 10e6 spleen cells were co-cultured with 2 10e6 irradiated (18000r) TC1 cells (E7 expressing tumor) for 7 days in the presenced or absence of ConA sup. ( 2%)

Target cells used to assess cytotoxicity were either Cr51 ( DuPont NEN 37MBq/ml) loaded (1hr at 37°C) TC1 cells or E7 pulsed EL4 cells (for 1 hr at 37°C during the Cr 51 loading of the cells10µg/ml of E7-derived peptide (49-57) (QCB) compared to EL4 cells NK dependant lysis was assessed on K562 target cells 2000 target cells were added / well of 96 well plate ( V botttom nunc 2-45128) with 100/1 being the highest Effector / target ratio. Controls for spontaneous or maximal Cr51 release were performed in sextuplet and were targets in medium or in triton 1.5%. All plates were gently centrifuged and incubated for 4 hrs at 37 in 7% CO2. 50 µl of the supernatant was deposed on 96w Lumaplate (Packard) let dry O/N and counted in a Top Count counter. Data is expressed as percent specific lysis which is calculated from the c.p.m. by the formula (experimental release - spontaneous release) / ( maximal release - spontaneous release) X 100.

**Serology**

Quantitation of anti E7 antibody was performed by Elisa using E7 (Bollen) as coating antigen. Antigen and antibody solutions were used at 50 µl per well. Antigen was diluted at a final concentration of 3 µg/ml in carbonate buffer ph9.5 and was adsorbed overnight at 4°c to the wells of 96 wells microtiter plates (Maxisorb Immuno-plate, Nunc, Denmark). The plates were then incubated for 1hr at 37°c with PBS containing 1% bovine serum albumin and 0.1% Tween 20 (saturation buffer). Two-fold dilutions of sera (starting at 1/100 dilution) in the saturation buffer were added to the E7-coated plates and incubated for 1 hr 30 min

at 37°c. The plates were washed 3 times with PBS 0.1% Tween 20 and biotin-conjugated anti-mouse IgG1, IgG2a or IgG2b or IgGtot (Amersham, UK) diluted 1/5000 in saturation buffer was added to each well and incubated for 1 hr 30 min at 37°c. After a washing step, streptavidin-biotinylated peroxydase complex (Amersham, UK) diluted 1/5000 in saturation buffer was added for an additional 30 min at 37°c. Plates were washed as above and incubated for 10 min with TMB( tetra-methyl-benzidine). The reaction was stopped with H2SO4 4N and read at 450 nm. Midpoint dilutions were calculated by SoftmaxPro (using a four parameters equation ).